Research Article

Promoting Effect of Ginsenoside Rb1 for GLUT-4 Gene Expression and Cellular Synthesis in C2Cl2 Muscle Cells

Seyed Ahmad Hosseini[1]*, Mohammad Reza Tabandeh[2] and Seyed Alireza Mesbah Namin[3]

[1]Nutrition and Metabolic Diseases Research Center Ahvaz Jundishapur, University of Medical Sciences, Ahvaz, Iran
[2]Department of Biochemistry and Molecular Biology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
[3]Department of Clinical Biochemistry, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
*E-mail address: seyedahmadhosseini@yahoo.com

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ABSTRACT

Ginseng is a most popular herb that its antidiabetic mechanisms have been studied for over a decade. Although stimulation of GLUT-4 redistribution in adipocyte and muscle cells has been described by different ginsenosides isolated from ginseng, however effect of ginsenoside Rb1 on the gene expression of GLUT-4 has not been reported so far. To investigate the effect of Rb1 on the GLUT-4 gene expression level we incubated the differentiated C2C12 myotubes with different concentrations of Rb1 (0.001, 0.01, 0.1, 1, 10 and 100 μM) for different times (1, 3, 6 and 12 hours).In general we found that Rb1 at all doses increased basal gene expression level of GLUT-4 in compared with untreated cells. Rb1 at doses of 1 and 0.1 μM and after 3hr had the maximal stimulatory effect. Results presented here help to better understand the mechanistic action of Rb1 ginsenoside on glucose uptake in muscle cells and it provides a rationale data for future applications of ginseng as a candidate for diabetes therapy.

Keywords: Ginsenoside Rb1, Upregulation, GLUT-4, Muscle cell
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INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a metabolic disorder that is characterized by high blood glucose due to insulin resistance and relative insulin deficiency (1) Patients with T2DM usually represent with metabolic syndrome and manifest disorder of carbohydrate and lipid metabolism(2). The overall prevalence of T2DM is about 6% of the world's adult population and rates of disease have increased markedly over the last 50 years in parallel with obesity(3).

Due to carbohydrate metabolism abnormalities, severe and chronic tissue damages are common in diabetic patient and achieving glucose control is important in reducing the complications of T2DM (2), (3).

In spite of insufficient evidence for herbal medicine safety and efficacy, many herbs have been used for centuries to manage blood glucose or improve health in diabetic patients(4). Among many herbal medicines, ginseng extract made from the root, rootlet, berry, and leaf of *Panax quinquefolium* (American ginseng) and *Panax ginseng* (Asian ginseng) is one of the most popular herbs that its antidiabetic mechanisms have been studied for over a decade(5).

The root of these *Panax* species contain more than 30 triterpene saponins mostly identified as ginsenosides (Rx) which are attributed to the most of ginseng's pharmacological activities(6).The data from analysis of *ginseng* roots

demonstrated that Rb1, Rb2, Rc, Rd, Re and Rg1 are the six major ginsenoside root extracts(7). Numerous reports showed that ginseng extracts can exert hypoglycemic and insulin sensitizing action and improve diabetic conditions in both humans as well as animals. Although mix crude ginsenosides have shown to have positive insulin sensitizing effects on fat and muscle at cellular level, whether a single component maximizes the therapeutic effect of ginseng on diabetes is not fully understood(8).

Glucose-stimulated insulin secretion, activating the insulin/IGF-1 signaling pathway and promotion of GLUT4 translocation have been described as molecular antidiabetic action of various ginseng components in different cell lines.

Ginsenoside Rb1 is one of the major constituents in ginseng root which its antidiabetic and insulin-sensitizing activities have been reported(9)'(10).The Rb1 stimulates glucose transport in adipose cells by promoting glucose-stimulated insulin secretion through protein kinase A and enhancement of insulin/IGF-1 signaling. Although ginsenoside Rb1 stimulates GLUT-4 translocation in adipose tissue, but the literature on the effect of this ginsenoside on the gene expression of GLUT-4 in adipose and muscle cells is currently unclear(10). In addition, many findings are based solely on the studies with cultured adipocytes, which lead us to the question of whether the ginsenoside Rb1regulates GLUT4 gene expression in other cell types.

This study aimed to identify the effects of ginsenoside Rb1 on the GLUT-4 gene expression in C2C12 myoblasts, in response to its different doses and exposure times, which has not yet been reported.

MATERIALS AND METHODS

2.1. Cell culture and differentiation
C2C12 myoblasts (CRL-1772) were obtained from the Pasteur Institute of Iran. C2C12 cells were maintained at 37°C and 10% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (D5796, Sigma Aldrich, Germany)supplemented with 10% fetal bovine serum (Gibco,10270-106,USA.) and 1% penicillin-streptomycin antibiotic (Bio West , L0018-100 , France). Media was changed every 24-36 hours. When the cells achieved 70% confluence, differentiation was induced by transferring myoblasts into differentiation medium consisting of DMEM with2% horse serum (Gibco, New Zealand) and 1% penicillin-streptomycin as described previously. Myotube formation was revealed by visualization of the cells under light microscopy after 24 hrs in differentiation medium. More than 90% of the cells expressed the myotube phenotype between 6 and 7 days after the initiation of differentiation and were used for the experiments.

2.2. Ginsenoside treatment
The myotubes used for the experiments were serum starved overnight in DMEM containing 0.2% BSA (Sigma Aldrich, Germany). Ginsenoside Rb1 (Phytolab, Germany, HPLC 99.9%) were dissolved in DMSO (less than 0.2% v/v) as a 1000-fold stock, and added to 0.2% BSA–DMEM at different final concentrations(0.001,0.01, 0.1, 1, 10 and 100 µM) and different lengths of time (1, 3, 6 and 12 hours). DMSO was present in thecontrol culture at similar concentration to ginsenoside treated cells about less than 0.2% (v/v).All assays were done as duplicate. Treated myotubes were trypsinyzed using 0.05% trypsin-EDTA (Gibco,27250-018,USA,) scraped in ice-cold PBS[(137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4 (pH 7.4)] and transferred into a 25-ml Falcon tube for centrifugation (700 g, 5 min, 4°C) to pellet intact cells.

2.3.RNA Isolation and Reverse Transcription
Total RNA was extracted from treated C2C12 myotubes using TriPure reagent according to the manufacturer's procedure (Roche, Germany), dissolved in diethyl pyrocarbonate (DEPC) treated water and quantified at a wavelength of 260 nm by nanodrop spectrophotometry (Eppendorf, Hamburg, Germany). The integrity of RNA was verified by optical density (OD) absorption ratio OD260nm/OD280nm between 1.8 and 2.0. For genomic DNA removal an in-solution DNase digestion was carried out by treating 1µg of RNA with 2 units of DNase I (Fermentas Inc, Vilnius, Lithuania).

First-strand cDNA was synthesized from 1µg of RNA using RocketScript RT PreMix kit (Bioneer Corporation, South Korea) and oligo dT following the manufacturer's instructions.

2.4. Quantitative Real Time PCR
A quantitative real-time PCR using qPCR™ Green Master Kit for SYBR Green I® (Jena Biosciense, Germany) was developed for detecting relative GLUT-4 levels in treated cells, in a ABI 7500 real-time PCR detection system (ABI, USA). Relative expression levels of GLUT-4 transcript were normalized to RNA loading for each sample using

GAPDH mRNA. Specific sets of primers (Macrogen, Seoul, South Korea) that were used for amplification of all genes were designed using Beacon Designer 7.1.

Sequences of sense and antisense, primers for GLUT-4 and GAPDH were as follows: for GLUT-4: 5'- ggg ctg tga gtg agt gct ttc-3' and 5'- cag cga ggc aag gct aga-3' and for GAPDH2: 5'- tgg tgg acc tca tgg cct ac.-3' and 5'- cag caa ctg agg gcc tct ct-3'.

Real time PCR reactions were performed using qPCR™ Green Master Kit in a final volume of 20 μL containing 3 μL DNAse treated cDNA. The reactions were performed with the following settings: 5 minutes of pre-incubation at 95°C followed by 40 cycles for 15 seconds at 95°C and 45 second at 60°C. Reactions were performed in triplicate. A reaction without cDNA was performed in parallel as negative control.

Relative quantification was performed according to the comparative 2-ΔΔCt method as described previously. The result for the gene expression was given by a unitless value through the formula 2-ΔΔCt. For analysis of qRT-PCR results based on ΔΔCt method Step OneTM software was used.

Validation of assay to check that the primer for the GAPDH and GLUT-4 had similar amplification efficiencies was performed as previously described.

2.5. Western Blot Analysis

C2C12 treated cells were lysed in 250 μl lysis buffer (225mmol Sucrose,4mmol Na2EDTA,20mmol HEPES.) at $4^{\circ C}$. 25 μl of the lysates were mixed with 25 μl Laemmli sample buffer supplemented with 2-mercaptoethanol at a final concentration of 7.5%(vol/vol). The samples were heated for 15 min at 65°C, separated by 7.5% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). The filters were blocked by incubation for 1 h in PBS with 5% nonfat milk. Blots were then washed in PBS-Tween and immunoblotted with the GLUT4 antibodies (Abcam, Cambridge, UK, Art No: 35826) (1:200 dilution). Detection of primary antibody was done using goat anti rabbit HRP-conjugated antibody (Abcam, Cambridge, UK, Art No:97265) (1:1000 dilution)and DAB reagent (Sigma Aldrich , Germany). Densitometric quantification ofprotein bands was performed using National Institutes of Health (NIH)Image J software.

2.7.Statistical analyses

Data were analyzed using the SPSS 14.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was used to test differences between various means followed by post hoc Tukey test. All experimental data were presented as the mean ± SD. The level of significance for all tests was set at $P < 0.05$.

RESULTS

In general incubation of skeletal muscle cells with Rb1 at different concentrations between 0.1-100 μM and different lengths of time (1-12 hr)resulted in significant induction of GLUT-4 gene expression in compared to untreated cells ($P<0.05$) (Fig. 1).

One hour incubation of skeletal muscle cells withRb1 at doses of 0.01 and 0.001 μM had no effect on the level of GLUT-4 gene expression level in relation to control, untreated cells ($P>0.05$) (Fig. 1). The highest level of GLUT-4 mRNA was observed after 1hr treatment of C2C12 myotubes with Rb1 at dose of 0.1 μM in relation to other cells those treated with Rb1 at doses of 100, 10 and 1 μM respectively ($P<0.05$) (Fig. 1).

When cultured cells were incubated with Rb1 for 3hr, maximal effect on the GLUT4 gene expression level was observed at doses of 10, 1 and 0.1 μM in relative to other treated cells ($P<0.01$) (Fig.2). Rb1 at dose of 0.001 μM had the lowest effect on GLUT-4 mRNA level after 3h of incubation in comparison with other treated groups ($P>0.05$) (Fig.2).

Incubation of C2C12 myotubes with Rb1 at a dose of 0.1 μM for 6hr resulted in a significant maximal induction of GLUT-4 gene expression in compared with cells those incubated with other concentrations of Rb1 ($P<0.05$) (Fig.3). Minimal effect of Rb1 after 6hr treatment was observed at doses of 0.001 and 100 μM respectively ($P<0.05$) in compared to other treated groups (Fig.3).

Rb1 at doses of 100, 0.01 and 0.001 μM at a time of 12 hr had the lowest stimulatory effect on the GLUT-4 transcript levels in C2C12 myotubes in relative to other concentrations ($P<0.05$) (Fig. 4). At this length of time Rb1 at concentrations of 1 and 0.1 showed significant maximal effect on the GLUT-4 mRNA level in compared with other doses of Rb1 ($P<0.05$) (Fig.4).
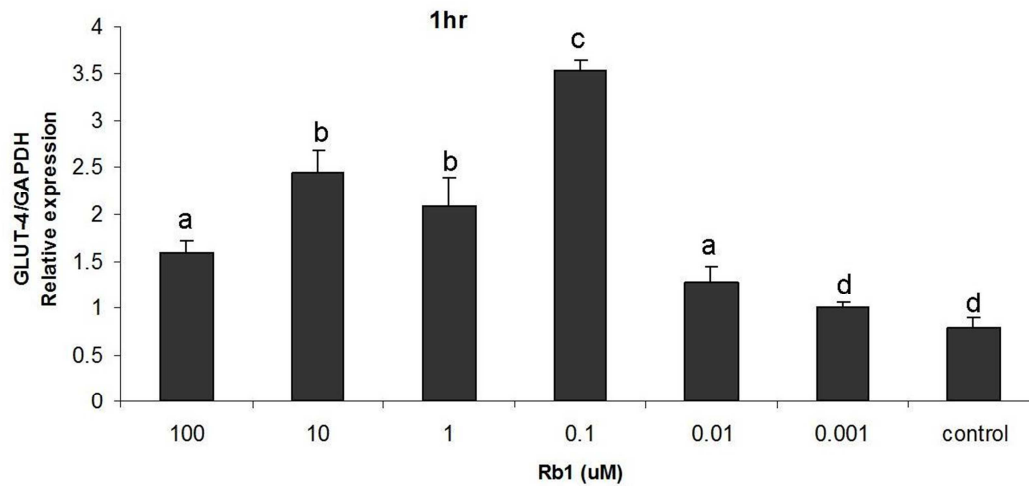
Fig.1. Relative gene expression of GLUT-4 in C2C12 cell after incubation with different doses of ginsenoside Rb 1 (0.001 – 100 μM) for 1hr

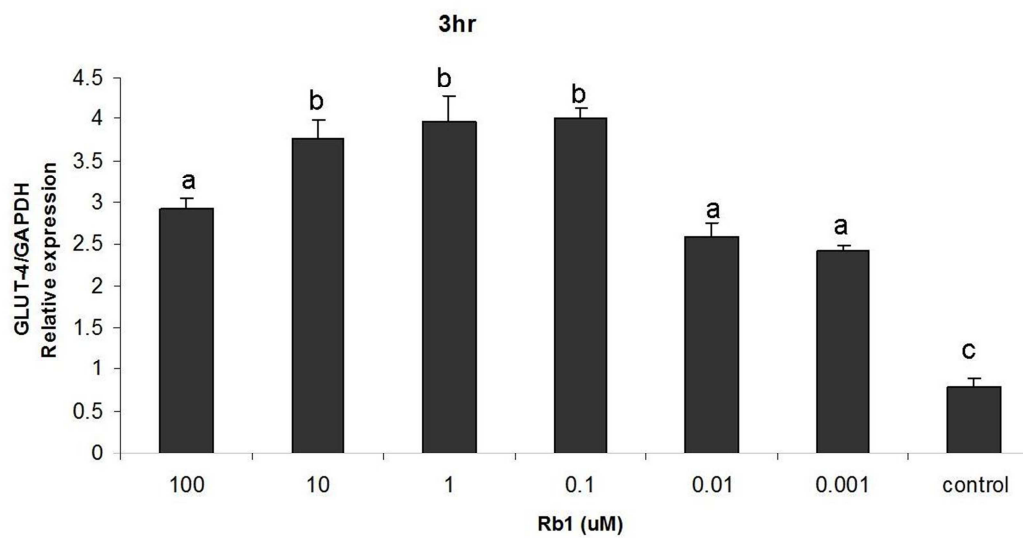*Different letters above each bar represent significant difference at p < 0.05.*

Fig.2. Relative gene expression of GLUT-4 in C2C12 cell after incubation with different doses of ginsenoside Rb 1 (0.001 – 100 μM) for 3hr

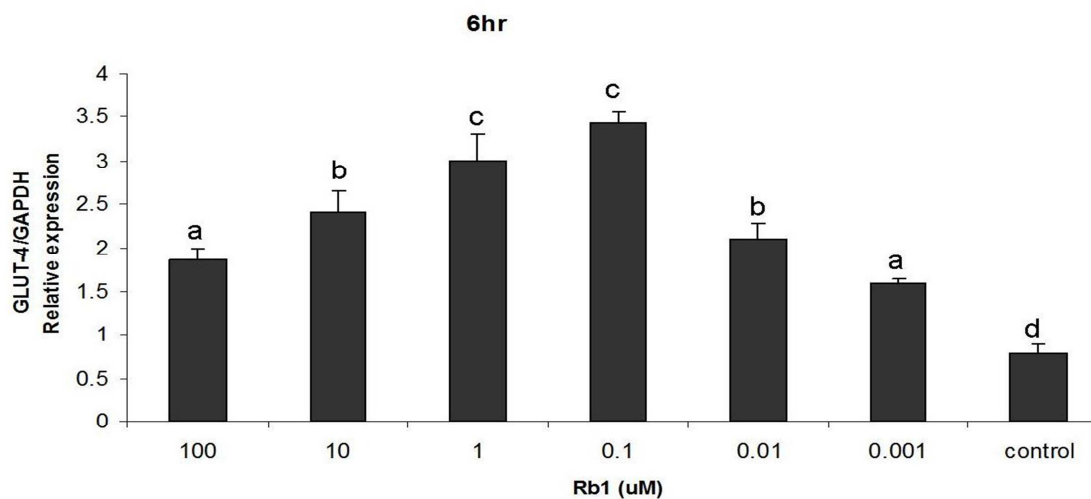*Different letters above each bar represent significant difference at p < 0.05*

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6hr

Fig.3. Relative gene expression of GLUT-4 in C2C12 cell after incubation with different doses of ginsenoside Rb 1 (0.001 – 100 µM) for 6hr

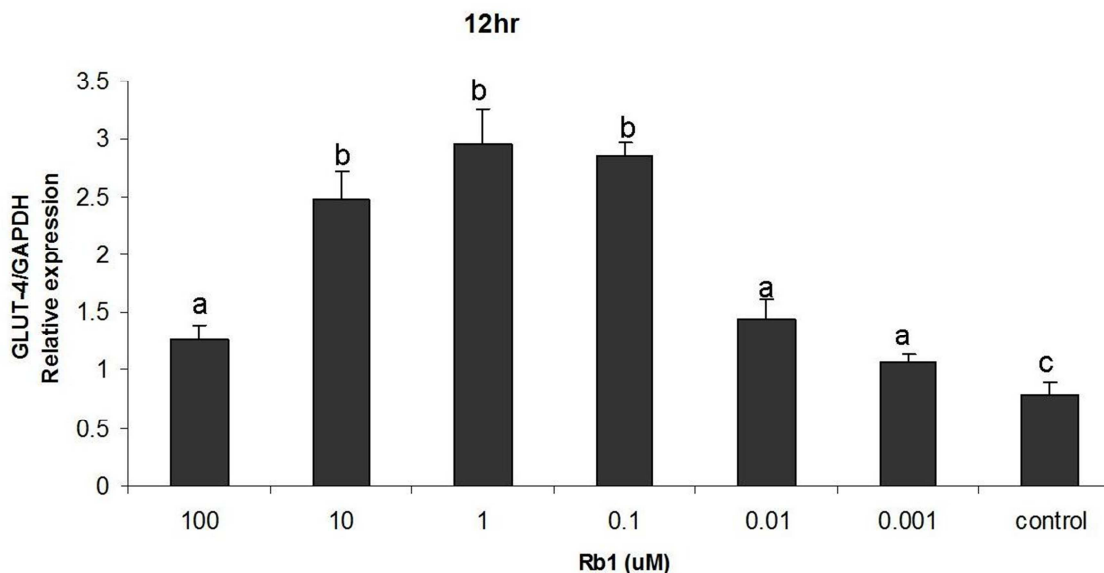*Different letters above each bar represent significant difference at p< 0.05.*

12hr

Fig.4. Relative gene expression of GLUT-4 in C2C12 cell after incubation with different doses of ginsenoside Rb 1 (0.001 – 100 µM) for 12hr

Different letters above each bar represent significant difference at p < 0.05.

As shown in figure 5 maximal GLUT4 protein level were significantly detected in the Rb1 treated cells at doses of 100, 1 and 0.1 µM in compared with that in the control untreated group.
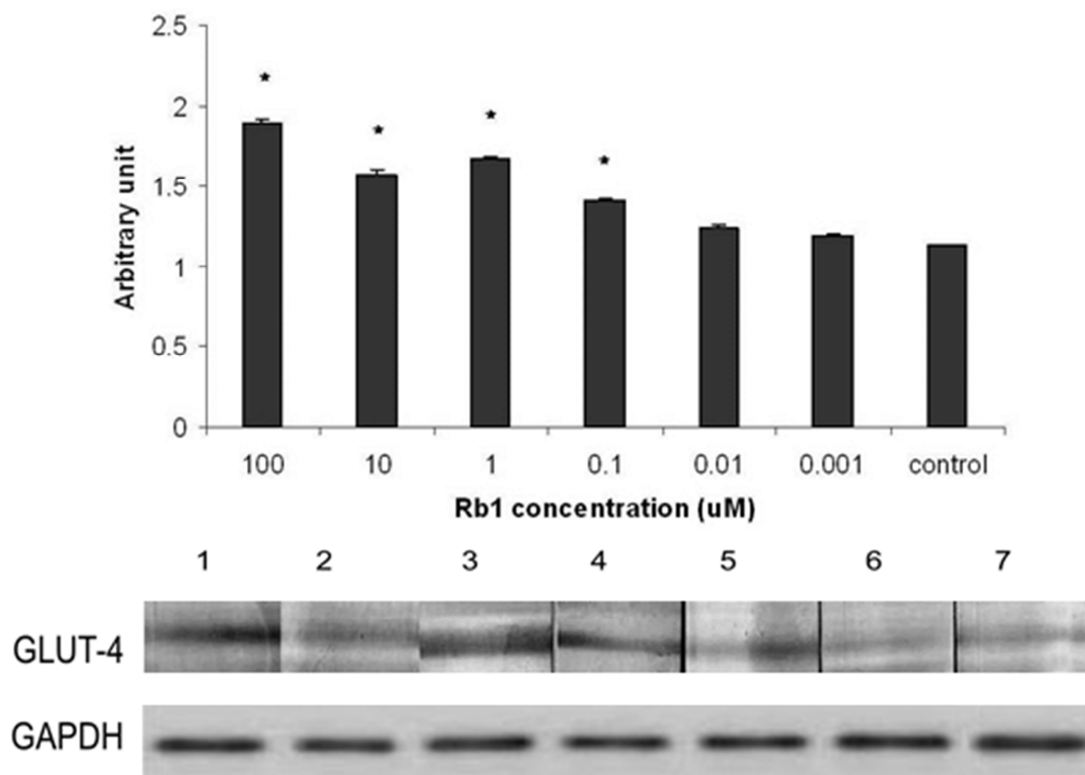
Fig. 5. Immunoblot analysis of GLUT4 protein expression in Rb1 treated C2C12 cells at different concentrations including 100 (lane 1), 10 (lane 2), 1(lane 3), 0.1 (lane 4), 0.1 (lane 5), 0.01 (lane 6) and in control, untreated cells (lane 7). The cells were collected 3 hr after incubation. GLUT-4 protein density quantization was done using JImage analysis software densitometer in comparison to GAPDH
Statistical significance was accepted at p <0.05.

DISCUSSION

Regulatory effects of different ginsenosides on the gene expression of GLUT-4 in adipocyte and muscle cells have not been reported so far. In the present study we showed that ginsenosides Rb1, one of the most abundant ginsenosides from Asian ginseng root, could stimulate GLUT-4 gene expression in C2C12 myotubes in a time and dose dependent manner.

Numerous studies have shown that different ginsenosides can stimulate basal and insulin dependent glucose uptake in insulin sensitive cells including adipocyte and muscle cells by different mechanisms (10)ʹ(10). One possible hypoglycemic activity of ginsenosides is stimulation of glucose transport across the cell membrane by enhancing the GLUT-4 translocation (10), (10), (11).

We observed that Rb1 at different doses from 0.001 to 100 μM increased basal gene expression level of GLUT-4 after different times of incubation from 1 to 12 hr in relative to untreated cells. Rb1 at doses of 1 and 0.1 μM had the maximal stimulatory effect on GLUT-4 mRNA level in C2C12 myocyte in comparison with other doses of Rb1. According to our findings, maximum concentration of GLUT-4 mRNA was observed after 3hr incubation of C2C12 myotubes with Rb1 at the most effective doses. Cells which treated with Rb1 at doses of 0.001 μM showed minimal elevation of GLUT-4 gene expression in compared with cells those received higher doses of Rb1.

We also found that exposure of C2C12 myotubes with different doses of Rb1 for longer time than 3 hr had no considerable effect on elevation of GLUT-4 transcript.

Different compounds extracted from leaf and root of ginseng have been reported to have anti diabetic effect (5). Certain ginsenosides such as Rb, Rg, Rh and Re are the major antidiabetic compounds of ginseng, in which different mechanisms have been described for their insulin sensitizing action at cellular and molecular levels (5), (8), (9),(12).

However, antidiabetic potency of these compounds is variable and tissue dependent according to their composition. For example Rg1, Rc, Rd, Re, Rf, Rg2, Rh1, Rb1, and Rb2 ginsenosides stimulate the glucose uptake in goat red

blood cells by increasing of glucose transporter 1, while Rg3 inhibited glucose uptake, and Rd, Ro, and Rh2 did not affect glucose uptake in these cells (13).

Stimulation of insulin signaling pathways and GLUT-4 translocation have been described as major mechanism of glucose uptake improvement and corresponding antidiabetic actions of ginsenosides in adipocyte and muscle cells. It has been shown that Rb1 can stimulate basal glucose uptake in adipocytes by enhancement of GLUT1 and GLUT4 translocations (10).

In another study, Rb1 promotes glucose-stimulated insulin secretion through protein kinase A, which augmented insulin receptor substrate 2 expression to enhance insulin/IGF-1 signaling in adipocyte (11). Jantunen et al reported that Re induces insulin receptor substrate-1 expression through the PI3K/Akt-PKC signaling cascade and stimulate GLUT-4 phosphorylation, resulting in glucose uptake enhancement in adipocyte.

Recent findings demonstrate that some ginsenoside increased expression of GLUT-4 due to an increase in beta-endorphin secretion and activation of opioid mu-receptors in fat and muscle tissue (14). Based on the above findings and our results we hypothesized that increasing of GLUT-4 translocation by Rb1 may be directly related to up-regulation of GLUT-4 gene in muscle cells. Some researches attempt to support this hypothesis which suggested that ginsenoside has a steroid skeleton in chemical structure and similar to dehydroepiandrosterone it may interacts with some G protein-coupled receptor, increases IRS-1 phosphorylation, GLUT-4 expression and glucose uptake (5), (6).

It seems that ginsenosides has beneficial actions for diabetes therapy. Although a mixture of components have shown to have positive effects in different cells, whether a single component like Rb1 has variable effect in different cells need to be clearer. Sufficient studies on the mechanism of action of ginsenosides help to practical applications of ginseng as a candidate for diabetes therapy. In conclusion, the results presented here clearly showed that Rb1 a major ginsenoside from ginseng up-regulates GLUT-4 gene expression in C2C12 myotubes; a mechanism which may indirectly increased the total numbers of GLUT-4 molecules that can translocate to the cell membrane and enhance glucose uptake.

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